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Antimicrobial Resistance and Resistance Gene Determinants in Clinical *Escherichia coli* from a Captive Population of Amur Tiger in China

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ABSTRACT

Antimicrobial resistance has emerged in the past few years as a major problem and many programs have been set up for its surveillance in human and veterinary medicine. The purpose of this study was to investigate the abundance and diversity of extended-spectrum β-lactamase genes and fluoroquinolone resistance genes from a captive population of Amur tiger in China. Most isolates were susceptible to amoxicillin/clavulanic acid, aztreonam, polymyxin B, and also exhibited high incidence rates of resistance to ampicillin, doxycycline, chloramphenicol, tetracycline and trimethoprim sulfur. Two gene cassette arrangements (dfrA12–aadA2 and dfrA15) were identified. The most prevalent extended-spectrum β-lactamase (ESBL) genes identified were TEM which was detected in 80% (24/30) isolates. The prevalence of quinolone resistance-determining region (QRDR) determinants (gyrA, gyrB, parC and parE) were investigated in a collection of 30 ESBL producing enterobacterial isolates with reduced susceptibility to fluoroquinolones. DNA sequencing revealed point mutations in gyrA (Ser83-Leu, Asp87-Asn, Glu 214-Gly), gyrB (Ser195→Asn), and parE (Ser85→ALa). No mutations were observed in parC. The epidemiological relationship between positive isolates was studied by pulsed-field gel electrophoresis (PFGE). PFGE analysis revealed these isolates were grouped into eleven clusters at 95% similarity level. We have demonstrated the distribution of the class I integrons and found gene cassettes not previously reported in integrons among *Escherichia coli* strains isolated from a captive population of Amur tiger.

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Authors' Contribution
YX and YH conceived and designed the study. YX and JC amplified and sequenced of antimicrobial resistance genes. WZ and SL performed pulsed-field gel electrophoresis. DL collected the clinical samples. YX, JC and WZ analyzed the data. YX wrote the article.

Key words
Extended-spectrum β-lactamase, fluoroquinolone, PFGE, antimicrobial susceptibility.

INTRODUCTION

Although antimicrobial therapy is used to treat clinical infections, antibiotic resistance in pathogenic bacteria from human, animal and environmental sources is recognized as a global problem in public health. *Escherichia coli* is considered as a member of the normal microflora of the intestine and is commonly found in the intestinal tract of animals and humans. It can also be implicated in animal and human infectious diseases. The increasing drug resistance in clinical isolates may be explained by antibiotic selection pressure and the widespread presence of integrons.

Integrons, which are widely distributed among bacteria, are capable of capturing, integrating antibiotic-resistance gene cassettes. Class 1 integrons are assumed to play an important role in the dissemination of antibiotic resistance genes. β-lactamases are an important cause of resistance to β-lactam antibiotics. Most extended-spectrum ß-lactamases (ESBLs) are derived from TEM- or SHV-type β-lactamases by one or more amino acid substitutions. Quinolones and fluoroquinolones are broad-spectrum antimicrobial agents extensively used in both human and veterinary medicine, and therefore found as residues in the environment. The most common mechanisms of resistance to quinolones in *E. coli* are mutations in DNA gyrase, such as topoisomerase II (in gyrA and gyrB) and topoisomerase IV (in parC and parE).

Captive populations of Amur tiger (*Panthera tigris altaica*) are companion animals that are in close contact with humans since ancient times, being possible the transference of bacteria between animals and humans. The Amur tiger is the largest of the five tiger subspecies. They are currently distributed mainly in the eastern mountainous areas of Heilongjiang and Jilin provinces in China, the Russian Far East and North Korea (Luan *et al*., 2011). Heilongjiang Amur Tiger Park is currently the world's largest wild natural garden for Amur tiger, which was established to save and protect some of the world's endangered species, including the Amur tiger. Currently, there is little information regarding the molecular basis of antibiotic resistance in *E. coli* strains isolated from the captive populations of Amur tiger, and therefore this study was carried out to screen and analyze the

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antimicrobial resistance in *E. coli* strains isolated from a captive population of Amur tiger in Heilongjiang Amur Tiger Park in China.

MATERIALS AND METHODS

Bacterial isolates

During May and November 2012, 61 *E. coli* strains were isolated consecutively from captive Amur tigers in Heilongjiang Amur Tiger Park. The fecal swabs were inoculated into nutrient broth (Oxoid TM, Thermo Fisher Biochemicals Ltd, Beijing 101312, China) and incubated at 37°C for 18 h, purified subsequently on MacConkey agar (Hangzhou Tianhe Microorganism Reagent Co. Ltd., Hangzou 310008, Zhejiang, China) and EMB (eosin methylene blue, Hangzhou Tianhe Microorganism Reagent Co. Ltd., Hangzou 310008, Zhejiang, China) agar plates, and incubated at 37°C for 24-48 h. For selective isolation and identification of *E. coli*, two blue-black colonies (presumptive *E. coli*) with a metallic sheen, growing on EMB agar plates, were randomly selected from each plate. *E. coli* ATCC 25922 was used as the reference strain. The strain was stored at -80°C until analysis.

Antimicrobial susceptibility testing

The antimicrobial sensitivity phenotypes of the *E. coli* strains were determined using a Kirby-Bauer disk diffusion assay, according to the standards and interpretive criteria described by Clinical and Laboratory Standards Institute guidelines. The antimicrobials including ampicillin, amoxicillin/clavulanic acid, cefalotin, aztreonam, gentamicin, kanamycin, apramycin, ofloxacin, ciprofloxacin, norfloxacin, enrofloxacin base, doxycycline, chloramphenicol, florfenicol, polymyxin B, trimethorpim sulfur and tetracycline were tested. All antibiotic disks were purchased from Oxoid (Thermo Fisher Biochemicals Ltd, Beijing 101312, China). Quality control for susceptibility testing was performed using *E. coli* ATCC 25922.

Characterization of antimicrobial resistance genes

The presence of genes encoding TEM, SHV, OXA and CTX-M β-lactamases were amplified by PCR in all ampicillin-resistant isolates using primers and conditions previously reported (Table I). The obtained DNA amplicons were sequenced on both strands and sequences were compared with those included in the GenBank database in order to identify the specific β-lactamase gene. In addition, the presence of gyrA, gyrB, parC and parE genes were studied for the quinolone-resistant isolates. DNA was extracted by boiling as above. The quinolone resistance-determining regions (QRDR) of gyrA, gyrB, parC and parE were amplified by PCR using the primers previously reported (Table I).

Pulsed-Field Gel Electrophoresis (PFGE)

Twelve isolates were subjected to PFGE using the restriction enzyme *Xba* I (New England BioLabs, Massachusetts, USA) according to the protocol described by PulseNet (Graves and Swaminathan 2001). Chromosomal DNA contained in agarose plugs was digested with 10 U of *Xba*I. PFGE was performed with a CHEF-DRIII system (Bio-Rad, Hercules, CA, USA), using an electric field of 6 V/cm at 14°C, angle of 120°C and switching times of 1.8-20s over 20 h. Migration of the DNA fragments was achieved in a 1.0% pulsed-field agarose gel (Bio-Rad)submerged in 0.5xTBE buffer (45 mmol/l Tris-Borate and 1 mmol/l EDTA) and *Salmonella Braenderup* H9812 was used a molecular reference marker. The gels were stained with Gel Red Acid Gel Stain (Biotum) and photographed.

RESULTS

Antimicrobial resistant E. coli

The antimicrobial susceptibility of 61 *E. coli* strains isolated from captive population Amur tigers to 17 antimicrobial agents are shown in Figure 1. About 70% isolates were susceptible to amoxicillin/clavulanic acid (73.66%), aztreonam (71.46%) and apramycin (71.71%). However, high resistance was shown against ampicillin (100%), tetracycline (85.12%), and trimethoprim sulfur (80.24%).

Antimicrobial resistance genes

Neither class 2 nor class 3 integrons were detected, while 52.46% (32 of 61) of the isolates were positive for the presence of intI1, which is a marker for class 1 integrons. Two distinct types of gene cassette array were characterized: dfrA15 and dfrA12–aadA2, respectively.

In this study, we identified 4 different types of ESBL genes: TEM (24), CTX-M (15), SHV (2) and OXA (6). TEM was the main type of β-lactamase among ESBL producing *E. coli* followed by CTX-M. The most prevalent extended-spectrum β-lactamase genes identified were TEM and CTX-M in this study.

The prevalence of QRDR determinants (gyrA, gyrB, parC and parE) was investigated in a collection of 30 ESBL producing enterobacterial isolates with reduced susceptibility to fluoroquinolones. DNA sequencing revealed point mutations in gyrA (Ser83→Leu, Asp87→Asn, Glu214→Gly), gyrB (Ser195→Asn) and parE (Ser85→ALa). No mutations were observed in parC.

Table I.- Primers for PCR reactions carried out in this study.

Primers	Sequences 5′→3′	Sizes (bp)	References
CTX-M-F	GGGCTGAGATGGTGACAAAGAG	905	Cormican *et al*. (1996)
CTX-M-R	CGTGCGAGTTCGATTTATTCAAC		
TEM-F	GTATCCGCTCATGAGACAATA	717	Cormican *et al*. (1996)
TEM-R	AGAAGTGGTCCTGCAACTTT		
SHV-F	TCTCCCTGTTAGCCACCCTG	593	Cormican *et al*. (1996)
SHV-R	CCACTGCAGCAGCTGCCGTT		
OXA-F	GCAGCGCCAGTGCATCAAC	198	Cormican *et al*. (1996)
OXA-R	CCGCATCAAATGCCATAAGTG		
GyrA-F	GAACTCACCCTTCCAGATCCA	982	Komp *et al*. (2003)
GyrA-R	GAGCGCGGATATACACCTT		
GyrB-F	GGACAAAGAAGGCTACAGCA	879	Komp *et al*. (2003)
GyrB-R	CGTCGCGTTGTACTCAGATA		
ParC-F	AGCGCCTTGCGTACATGAAT	964	Komp *et al*. (2003)
ParC-R	GTGGTAGCGAAGAGGTGGTT		
ParE-F	GACCGAAAGCTACGTCAACC	958	Komp *et al*. (2003)
ParE-R	GTTCGGATCAAGCGTGGTTT		
IntI1-F	F:CCTCCCGCACGATGATC	280	Shaheen *et al*. (2010)
IntI1-R	R:TCCACGCATCGTCAGGC		
IntI2-F	F:TTGCGAGTATCCATAACCTG	288	Shaheen *et al*. (2010)
IntI2-R	R:TTACCTGCACTGGATTAAG		
Int-F	F:GGCATCCAAGCAGCAAG	Variable	Shaheen *et al*. (2010)
Int-R	R:AAGCAGACTTGACCTGA		

F, forward; R, reverse

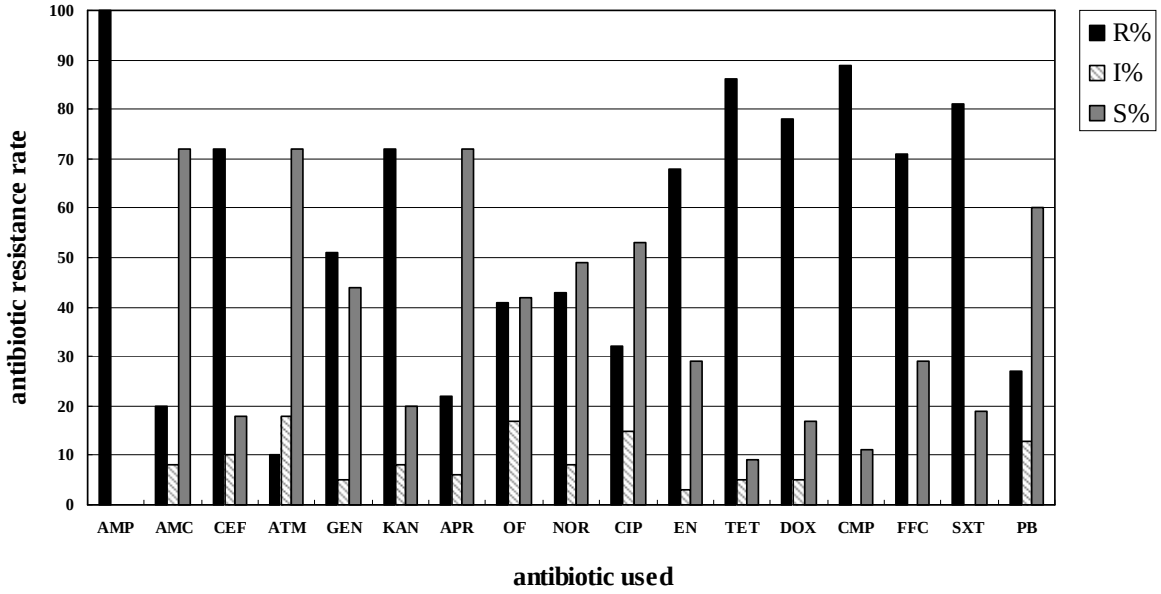

Fig. 1. *In vitro* susceptibilities of 61 *E. coli* strains isolated from captive Amur tiger to 17 antimicrobial agents. R%, resistance rate; I%, intermediate rate; S%, susceptible rate; AMP, ampicillin; AMC, amoxicillin/clavulanicacid; CEF, cefalotin; ATM: aztreonam; GEN, gentamicin; KAN, kanamycin; APR, apramycin; OF, ofloxacin; NOR, norfloxacin; CIP, ciprofloxacin; EN, enrofloxacin base; DOX, doxycycline; CMP, chloramphenicol; FFC, florfenicol; SXT, trimesulf; PB, polymyxin B; TET, tetracycline.

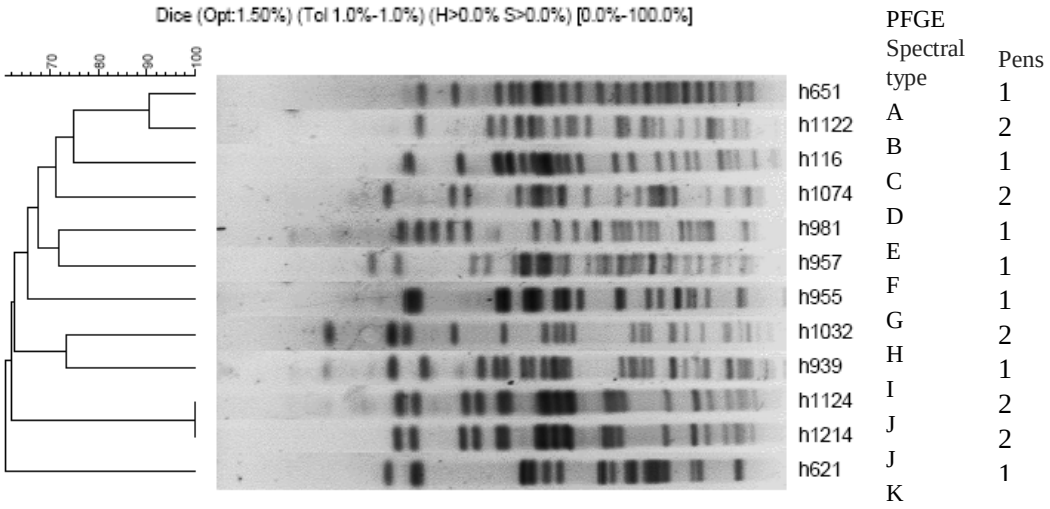

Fig. 2. Fingerprints and dendrogram obtained by PFGE of 12 *Escherichia coli* strains isolated in the present study.

Relationship between positive isolates

PFGE of *Xba*I-digested genomic DNA from 12 *E. coli* isolates showed eleven different macrorestriction profiles, each corresponding to a different serovar. No differences were observed between PFGE profiles of *S. Montevideo* when isolates from different tiger droppings were compared (Fig. 2). Fingerprints and dendrogram obtained by PFGE of 11 *E. coli* strains isolated in the present study revealed similar pattern suggesting the possibility of circulation and transmission of clones of limited diversity in our region.

DISCUSSION

Antimicrobial susceptibility patterns of *E. coli* isolates to 17 antimicrobial agents were obtained in this study. Most of the isolates were susceptible to amoxicillin/clavulanic acid, aztreonam and apramycin. On the other hand, the high incidence rates of resistance to tetracycline, chloramphenicol and trimethoprim sulfur may be derived from the frequent, long-term and widespread use of these antimicrobials in the park studies. In the multiply resistant strains, the rates of resistance of *E. coli* to ciprofloxacin, norfloxacin and ofloxacin were similar.

In this study, the prevalence of integron–positive isolates was 52.46%, which is similar to the data obtained from the Beijing area in China, which showed a prevalence of 54.70% (Du *et al*., 2005). It has been indicated that the prevalence of integrons is related to the antimicrobial pressure in the environment. The investigation of resistance gene cassettes in this study revealed that aminoglycoside resistance determinants (aadA2) and trimethoprim resistance determinants (dfrA15) were prevalent among *E. coli* strains isolated from a captive population of Amur tiger. We have not found any previous reports in the scientific literature on integrons and associated gene cassettes in *E. coli* or other bacterial pathogens from the captive population of Amur tigers in Heilongjiang Amur Tiger Park. Similar to our study, class 1 integrons, including dfr and aadA gene cassettes, have been found to be the most prevalent type of integron in *E. coli* isolates from humans, animals and food in other studies.

ESBLs represent an important mechanism of resistance in Enterobacteriaceae. In recent years, ESBL producing strains have been reported more and more frequently in China (Xiong *et al*., 2004). The β-lactam antibiotics are inactivated by Beta-lactamases that hydrolyze the amide bond that exists in the beta-lactam ring, disrupting the ring structure and make the antibiotics nonfunctional against bacteria (Khan *et al*., 2014). In recent years, the prevalence of ESBLs-producing *E. coli* is increasing and the resistance becomes more and more serious (Xue *et al.*, 2012). In this study, we determined 24 *E. coli* isolates were the prevalent TEM–type ESBLs. TEM was the main type of β-lactamase, and CTX-M was the second. SHV was detected in 2 isolates, and OXA was detected in 6 isolates.

Fluoroquinolones are highly effective road-spectrum bantimicrobials for treatment of a variety of clinical and veterinary infections. Fluoroquinolones resistance in bacteria can be due to chromosomal and plasmid mediated mechanisms. In this study, DNA sequencing revealed point mutations in gyrA, gyrB and pare, but not in parC. Quinolone resistance has been reported in *E. coli* isolated from retail chicken products and from healthy and clinically affected birds (Johnson *et*

al., 2005). An increase in the prevalence of fluoroquinolone resistance is therefore unexpected and strongly undesirable.

In conclusion, we have demonstrated the distribution of the class I integrons among *E. coli* strains isolated from a captive population of Amur tiger in Heilongjiang Amur Tiger Park. We found gene cassettes not previously reported in integrons among *E. coli* from this captive population of Amur tiger. The genetic content, combination and frequency of emergence of these gene cassettes may reflect the antibiotic selection pressure in Heilongjiang Amur Tigers Park, China, and provide useful surveillance information for the rational use of antimicrobials. The potential role of integrons in the uptake and dissemination of resistance genes may be a continuing threat to the effectiveness of certain antibiotic therapeutic agents in Amur Tiger Park.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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